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Ureteral obstruction decreases renal prepro-epidermal growth factor and Tamm-Horsfall expression

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Ureteral obstruction decreases renal prepro-EGF and Tamm-Horsfall expression. Northern and dot-blot analysis of polyadenylated RNAs of kidney cortical and outer medullary tissue was performed in male Sprague-Dawley rats at varying times up to 24 hours after bilateral ureteral obstruction (BUO), after 24 hours of unilateral obstruction (UUO) and at varying periods after release of BUO or UUO. Pre-proEGF (preproEGF) and Tamm-Horsfall (TH) mRNA declined by four hours of BUO to virtually undetectable levels at 24 hours of ureteral obstruction. Upon release of BUO or UUO, preproEGF and TH mRNA returned slowly toward normal but remained below control levels up to four days after release of ureteral obstruction. Urinary EGF excretion paralleled these changes in renal preproEGF mRNA. Although these changes are similar to those observed during nephrotoxic and ischemic renal failure, where the expression of the immediate early genes precedes the fall in preproEGF and TH expression, no such increase in the expression of these genes occurred after bilateral ureteral obstruction. These changes in preproEGF and TH expression could also be dissociated from uremia and high rates of DNA synthesis, suggesting that ureteral obstruction itself is a sufficient cause of the reduced expression. The increase in ureteral pressure and its functional and humoral effects may each play a role in reduced preproEGF and TH expression during ureteral obstruction.

Recovery from acute renal failure is associated with increased renal nucleic acid synthesis and renal cell mitosis [1–3]. Increased immediate early gene expression precedes these events and their immediate but short-term expression is very similar to that observed when cells enter the growth cycle after stimulation by serum or specific growth factors [3]. Thus it seems likely that the expression of the immediate early genes by the kidney after renal ischemia is related to the process of cell growth and division. By contrast, renal ischemia and cisplatin-induced renal failure reduce the expression of two prominently expressed genes in the kidney, prepro-epidermal growth factor (preproEGF) and Tamm-Horsfall [3–5] and unpublished observations). While the reduced expression of these genes occurs before the increase in DNA synthesis, reduced expression remains low throughout and beyond the period of active DNA synthesis.

To date, this decline in renal preproEGF and TH expression has been studied only in models of acute renal failure associated with prominent degrees of cell necrosis and active rates of DNA synthesis. Thus the repression of these two genes may be linked to the commitment to cell division. To determine whether reduced renal preproEGF and Tamm-Horsfall expression could be dissociated from high rates of renal DNA synthesis we performed experiments in rats in which acute renal failure was induced by complete ureteral obstruction. Renal cell necrosis, enhanced DNA synthesis and regeneration are not prominent in this model of acute renal failure [6]. The results show that ureteral obstruction reduces preproEGF and TH gene expression coordinately without elevated immediate early gene transcription, cell necrosis or high rates of DNA synthesis. Obstruction itself is responsible for these changes in gene expression.

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Methods

Animal preparation

BUO. Rats undergoing obstructive renal failure reduce food intake and lose weight. For this reason our studies were performed under conditions of paired feeding to prevent any differences in renal mRNA measurements secondary to differences in food intake. All experiments were performed in Sprague-Dawley rats weighing 250 to 500 g, housed in metal metabolic cages fitted with fine mesh screens. Each animal was offered 30 grams of a standard rat chow (Rat Chow 5012, Ralston Purina Co., St. Louis, Missouri, USA, containing 22.8% protein, 4.5% fat, 4.6% fiber and water) and 50 ml of tap water daily. After a period of four to six days, when they ate their allotted portions of food, rats were anesthetized (ketamine, 75 mg/kg) and, under sterile conditions, both ureters occluded with metal removable surgiclips (5 mm) placed around each ureter with its surrounding connective tissue and fat. The period of BUO varied up to 24 hours in duration and only rats who were totally anuric during the period of BUO were studied. Animals were sacrificed after varying periods of BUO without release, or reoperated on 24 hours later to remove the clips for studies after relief of obstruction.

UUO. Animals treated as above underwent unilateral ureteral obstruction (UUO) and were sacrificed either after 24 hours without relief of obstruction or at varying times after relief of 24 hours UUO.

Control. Animals underwent sham operations and were pair fed to control for changes induced by surgery and food intake. In sham operated animals, anesthesia, manipulation of the renal hila, and exposure of the peritoneal cavity was performed as in the obstructive studies, but obstruction of the ureters was omitted.

The daily consumption of food and water was measured and urine was collected under oil in plastic cylinders to which thymol was added. Each day the rats were weighed and the 24-hour urine volume measured. A portion of the urine was frozen and stored at −20°C for later analysis of osmolality and EGF concentration. Blood was taken by cardiac puncture under anesthesia at varying time periods of BUO or UUO and one to six days after relief of BUO. Animals were sacrificed at similar intervals as required by the specific protocols.

RNA blot analysis

Relative levels of mRNA were analyzed by Northern analysis in at least two animals for each condition studied. Total cortical and outer medullary RNA was isolated from anesthetized animals at varying time periods after obstruction and release or sham treatment by the guanidinium thiocyanate procedure [7] after intraarterial perfusion of the kidney with cold buffered saline (154 mM NaCl, 50 mM Tris, pH 7.4). Poly(A^+) RNA was isolated by oligo(dT)-cellulose (Type 3, Collaborative Research, Bedford, Massachusetts, USA) chromatography [8]. Poly(A^+) RNA (5 μg/lane) was either electrophoresed through formaldehyde-1% agarose gels [9] and transferred overnight to nitrocellulose membranes (BA 85, Schleicher and Schuell, Inc., Keene, New Hampshire, USA) as described [10], or applied directly (1 μg/lane) to the membranes by filtration. Hybridization was carried out with nick translated DNAs labeled with ^{32}P-dCTP (500 Ci/mmol) to a specific activity of approximately 5×10^8 cpm/μg DNA [11]. Nitrocellulose blots were prehybridized at 45°C overnight with denatured salmon sperm DNA (0.1 mg/ml) in 5 × SSC, 50% deionized formamide, and 0.02% Denhardt's solution (0.02% ficoll, 0.02% polyvinylpyrrolidine, and 0.02% BSA) and hybridized at 45°C overnight in the same solution containing 10% dextran sulfate and radiolabeled probe. Non-specifically bound radioactivity was removed by extensive washing as described [12]. After washing, the blots were air dried and exposed overnight to Kodak XAR film at −70° with intensifying screens. Quantitative variabilities of isolation and transfer of RNAs were accounted for by reprobing the Northern blots with a cDNA probe for glyceraldehyde-3-phosphate-dehydrogenase. The sequences used for nick translation were excised with appropriate restriction endonucleases and purified by preparative gel electrophoresis. The cDNAs for murine preproEGF (a 700 bp Pst I fragment of pmegf10, a gift of G. Bell [13]); TH (a 2.2 kb TH Eco R1 fragment in pBR322, a gift of D.V. Goeddel [14]; and rat glyceraldehyde-3-phosphate-dehydrogenase from prGAPDH-13, a gift of J.M. Blanchard [15]). To quantify levels of preproEGF mRNA, autoradiograms were scanned in two dimensions using an Apple scanner and densitometry employing 256 gray scales on an Apple MacIntosh IIcx computer using Image 1.36 software (Wayne Rasband, National Institutes of Health).

^{3}H-thymidine incorporation

Studies were performed in 10 BUO and six sham operated rats after 24 hours of obstruction. One hour after i.p. ^{3}H-methyl thymidine (0.5 μCi/g body wt), animals were anesthetized, the kidneys flushed with 0.9% NaCl intraarterially, and removed. Portions of the entire kidney were homogenized in 40 volumes of 150 mM NaCl, 100 mM EDTA pH 8. SDS was added to a final concentration of 1% and mixed gently. $NaClO_4$ was added to a final concentration of 1 M. The mixture was extracted 1:1 with phenol:isoamyl alcohol:chloroform (1:1:24) and spun at 5000 rpm for 10 minutes. Ninety-five percent ethanol was then added to the supernatant and with gentle stirring the precipitated DNA removed with a glass rod. After washing with 75% ethanol, the precipitate was dissolved in 100 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA (STE) at 37°C and treated with DNase free RNase A (20 μg/ml) for one hour at 37°C. The DNA was reprecipitated and suspended in STE and stored at 4°C for further analysis.

Analytical methods

Urine samples were assayed for EGF receptor-binding activity in duplicate using membranes from A431 cells as the source of receptors and highly purified mouse EGF as ^{125}I-labeled tracer and reference standard (Biomedical Technologies, Stoughton, Massachusetts, USA). A standard curve using known concentrations of unlabeled mouse epidermal growth factor reference standard was included in each assay. Blood urea nitrogen was measured using the diacetyl monoxime method [16]. ^{3}H activity in urine, plasma, tubule fluid and isolated DNA was determined in a liquid scintillation spectrometer (Packard Tri-Carb model 2425, Downers Grove, Illinois, USA). The concentration of DNA was determined by measuring its absorbance at 260 μm.

Results

The effect of bilateral ureteral obstruction on blood urea nitrogen is shown in Figure 1. The BUN rises progressively through 24 hours of BUO to a peak value of 123.3 ± 14.1 mg%. Following release BUN falls to 38.5 ± 5.4 mg% at 24 hours and to near control values by four days post-release. Thus 24 hours of BUO and release is a partially reversible form of acute renal failure.

The results of the studies of renal DNA ^{3}H-thymidine incorporation are summarized in Figure 2. DNA synthesis was not increased by bilateral ureteral obstruction of 24 hours duration when BUN values had peaked. Similar measurements during renal ischemia, by contrast, reveal increases in values of ^{3}H-thymidine incorporation that reach a peak that coincides with maximal BUN values [3]. This difference cannot be explained by differences in kidney content of ^{3}H-thymidine (unpublished observations).

Northern analysis of kidney mRNA are summarized in Figure 3. Renal preproEGF (panel A) and TH mRNAs decreased beginning at four hours of BUO and were not detectable after 12 hours of BUO. PreproEGF and TH mRNAs were also markedly reduced after 24 hours of UUO. In studies of the long term effects of BUO (Fig. 4), Northern blot analysis revealed that although 24 hours of BUO reduced renal preproEGF expression to virtually undetectable levels these levels increased progressively when the ureteral clamp was removed. Dot blot analysis

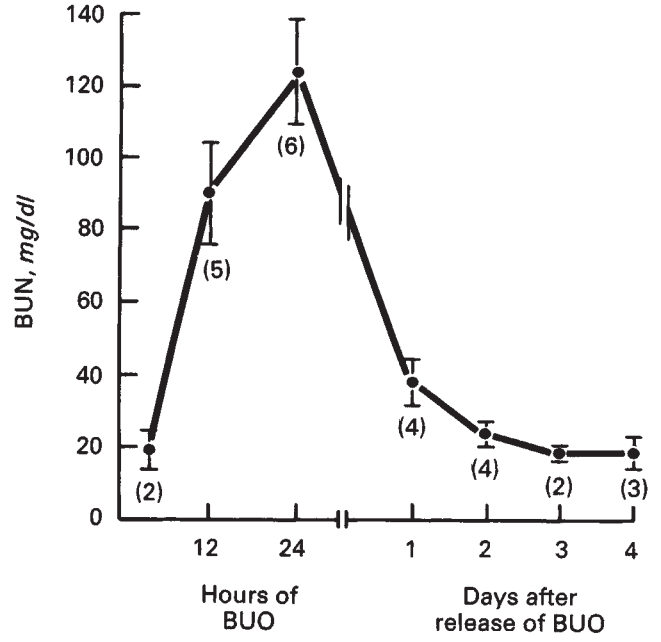

Fig. 1. *Blood urea nitrogen (BUN) in rats during and after relief of bilateral ureteral obstruction.* Number in parenthesis is number of rats.

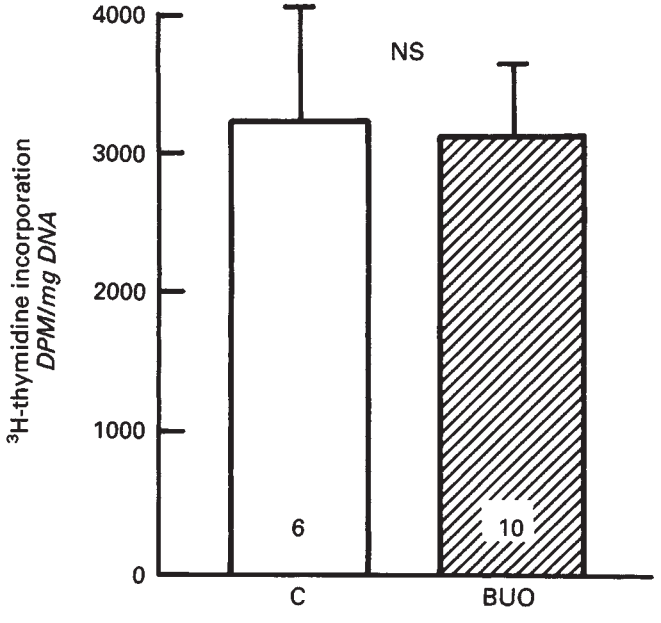

Fig. 2. *Renal DNA synthesis in sham treated animals and in animals 24 hours after bilateral ureteral obstruction.* The numbers within the bars indicate the number of animals studied.

of kidney mRNA using the Tamm-Horsfall cDNA (Fig. 5) shows a similar progressive increase in Tamm-Horsfall mRNA with release of the ureteral obstruction. By contrast, sham operation itself did not effect renal preproEGF or TH expression as mRNA levels for each showed the same proportion of GAPDH mRNA [preproEGF 88 ± 0.3%, N = 4 (unoperated) and 108 ± 23%, N = 8 (sham operated), NS; TH 60 ± 19%, N = 5 (unoperated) and 60 ± 12%, N = 5 (sham operated), NS].

Daily urinary excretion of EGF was measured at varying times after the release of 24 hours BUO and the results are summarized in Figure 6. Urinary EGF excretion fell from mean values of 13.5 ± 1.1 μg/day before ureteral obstruction to 0.9 ± 0.2 μg/day during the first 24 hours after release of the ureteral clamp. Daily EGF excretion increased progressively but to only 8.8 ± 2.0 μg/day by the fourth day after release, and significantly below the control levels.

Additional studies were performed to determine the response of renal preproEGF and TH expression to UUO and subsequent release. Figure 7 summarizes the results of Northern analysis of renal preproEGF and GAPDH mRNA and shows marked reduction of preproEGF mRNA after 24 hours of UUO and a gradual return of preproEGF mRNA to 58.4 ± 13.8% (N = 3) of control values four days after release of 24 hours of UUO. Similarly, Northern analysis of TH mRNA (Fig. 8) shows that TH mRNA falls markedly after UUO and returns toward normal by four days after release of UUO (82.5 ± 9.8% versus control, N = 2).

Discussion

We have previously established the interrelationship between acute renal failure and decreased preproEGF and TH mRNA synthesis [3, 5]. These models of ARF were coincident with renal cell necrosis and regeneration. We wished to investigate whether the decrease in mRNA levels was dependent on either cell necrosis or the commitment to increased DNA synthesis. The results of the present studies demonstrate that renal cell necrosis is not a necessary precondition for reduced renal expression of preproEGF and Tamm-Horsfall. Neither enhanced renal DNA synthesis nor prominent expression of the immediate early genes were demonstrated after bilateral ureteral obstruction, yet renal mRNA for preproEGF and TH fell markedly. The results of the present studies would suggest that the decline in preproEGF mRNA is not sufficient by itself to signal enhanced renal DNA synthesis.

The decline in expression was related to the length of ureteral obstruction. PreproEGF and TH mRNA declined initially at four hours of obstruction and were undetectable at 12 hours of obstruction. This time course is quite similar to the decline in preproEGF and TH mRNA after release of 50 minutes of renal hilar clamping. As in ischemia, the return of renal EGF production is slow, as renal preproEGF mRNA and EGF excretion are well below pre-obstruction control values four days after release (Figs. 4 and 5). Also, and similar to renal ischemia, the decline in preproEGF and TH mRNA was the same whether ureteral obstruction was unilateral or bilateral, eliminating a prominent role for uremia in the decline in renal EGF production. Taken together these data are consistent with the notion that ureteral obstruction reduces preproEGF and TH expression by a direct effect of obstructing the ureters.

Previous studies show that ureteral obstruction has diverse effects on thick ascending limb and distal tubule, the sites of synthesis of both preproEGF and TH [17, 18]. Ureteral obstruction increases intraluminal pressure [19], reduces transepithelial NaCl transport [20] and Na-K ATPase levels [21] in the thick ascending limb. On the other hand, ureteral obstruction increases thick ascending limb H-ATPase activity [21]. Thus obstruction causes both positive and negative effects on thick ascending limb synthetic and metabolic function. The present

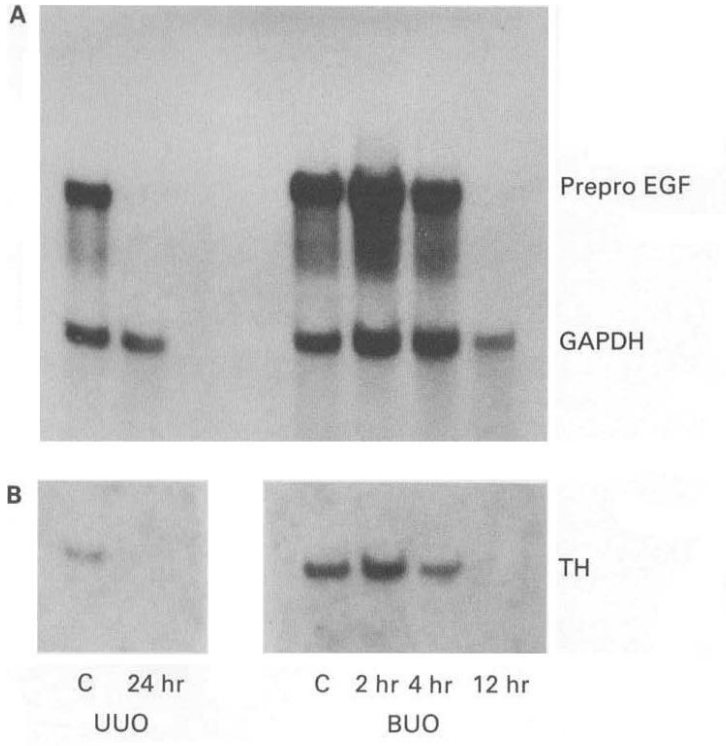

Fig. 3. *A. Northern analysis of renal preproEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA at varying times after unilateral (UUO) or bilateral (BUO) ureteral obstruction.* Five micrograms of poly(A^+) RNA per lane. **B.** The blot from panel A was reprobed with a Tamm-Horsfall (TH) cDNA.

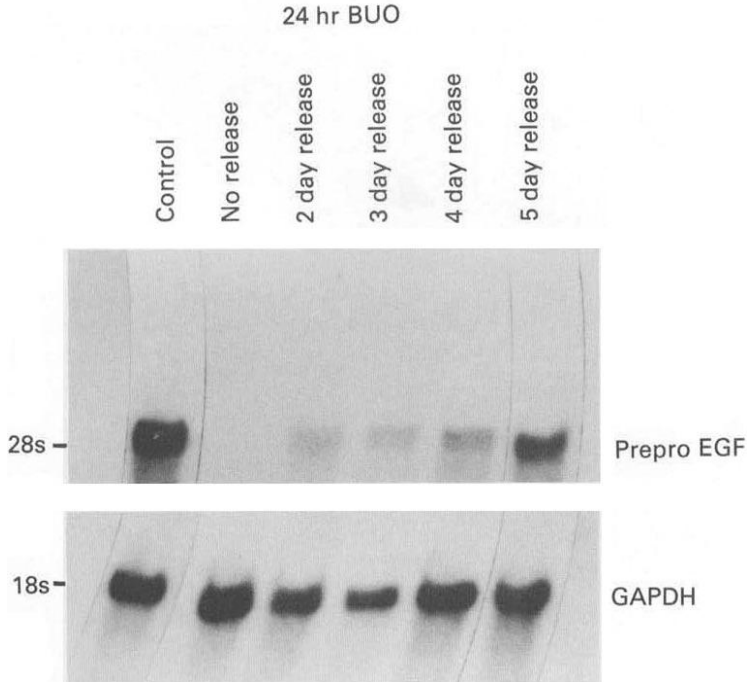

Fig. 4. *Northern analysis of renal preproEGF and GAPDH mRNA in control (lane 1) and in animals with 24 hours of BUO without release (lane 2); and in animals 2 days (lane 3); 3 days (lane 4); 4 days (lane 5); and 5 days (lane 6) after release of 24 hours BUO.* The migration of 18s and 28s RNA is indicated on the left.

studies further demonstrate that some of these effects of obstruction on the thick ascending limb occur at the level of gene expression.

The molecular mechanism of reduced expression was not explored and whether ureteral obstruction reduces preproEGF and TH mRNA transcription or reduces mRNA stability will have to be determined. Reduced glomerular filtration rate and thick ascending limb salt transport, as well as increased intratubular pressure occur in either the ischemic or obstructive forms of acute renal failure and suggest that these functional changes may somehow play a role in the reduction in preproEGF and TH expression. Ureteral obstruction has marked effects on three prominent intrarenal hormone systems: the metabolism of arachidonic acid, the renin-angiotensin system, and the renal kallikrein-kinen system [22], so that interactions between these systems and EGF are possible. As bilateral ureteral obstruction

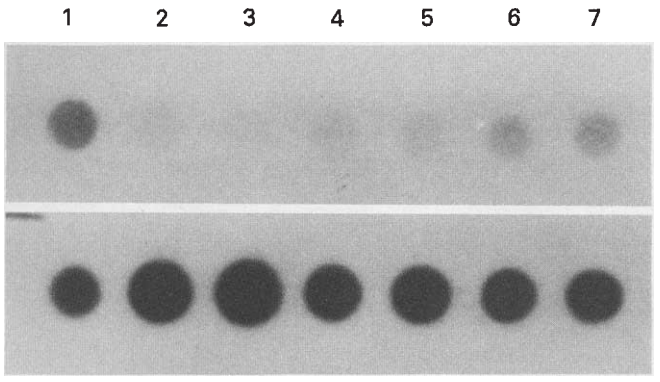

Fig. 5. *Dot-blot analysis of TH and GAPDH mRNA (1 μg/lane) from male Sprague-Dawley rat kidneys 24 hours after sham-operation (lane 1), 24 hours after BUO (lane 2), and one (lane 3), two (lane 4), three (lane 5), four (lane 6), and five days (lane 7) after relief of obstruction.* Individual blots were hybridized with probes specific for Tamm-Horsfall (**A**), or GAPDH (**B**).

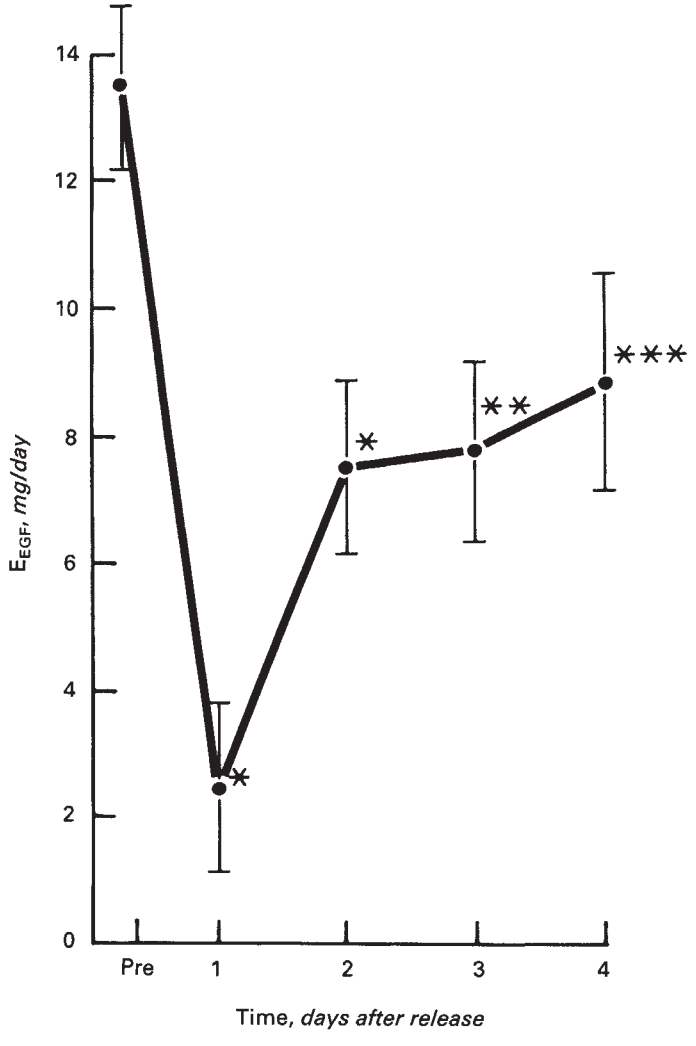

Fig. 6. *Daily urinary EGF excretion before (Pre) and after release of 24 hours BUO.* $*P < 0.001$ compared to Pre-value; $**P < 0.025$; $***P < 0.05$, paired *t*-test. Control urinary EGF excretion was not different from the PRE values and did not change significantly after surgery (data not shown).

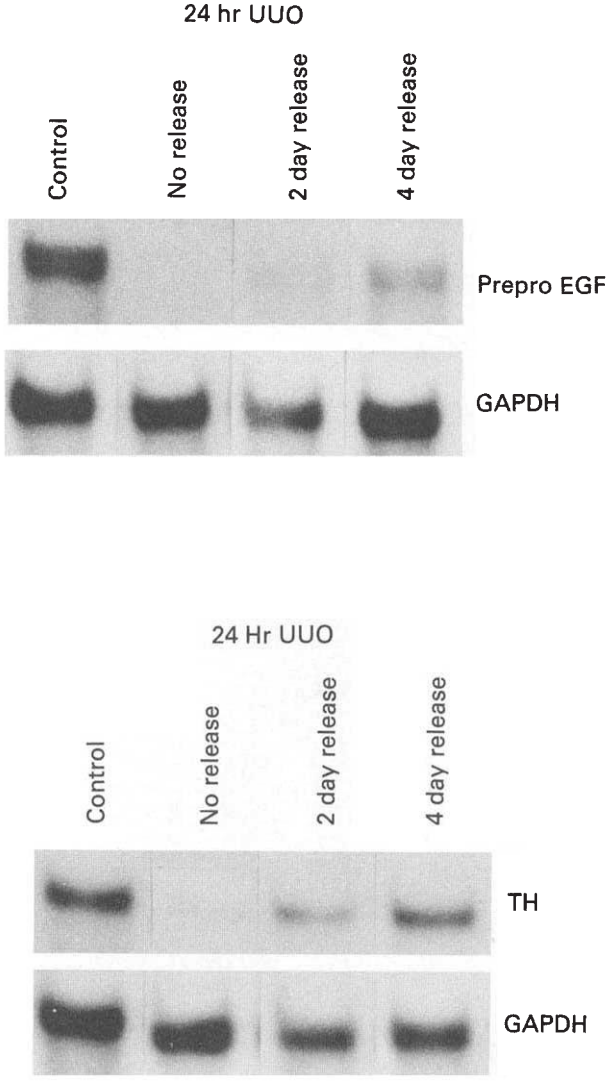

Fig. 8. *Northern analysis of renal TH and GAPDH mRNA in control animals (lane 1) and in animals with 24 hours of UUO without release and in animals at varying times after release of 24 hours of UUO.*

increases systemic ANF levels but unilateral ureteral obstruction does not [23], interaction between ANF and EGF seems unlikely. Additional studies will be necessary to pinpoint the level at which obstruction affects the renal expression of preproEGF and TH.

The possible role of reduced renal production of EGF and TH

in the abnormal function of the kidney during obstruction is of course unknown. It is interesting to note that both preproEGF and TH increase post-natally [18, 24] so that interruption of this pattern of expression could in part be responsible for the severe consequences of prolonged ureteral obstruction on renal function and morphology, especially in the neonatal period [25]. Cloning of the rat preproEGF gene [26] should provide an important opportunity to determine the mechanism by which obstruction affects renal gene expression.

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